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Abstract: Background There are emerging reports that the family of a disintegrin and metalloproteinases (ADAM) are involved in the maintenance of the malignant phenotype of glioblastomas. Notably, ADAM proteases 10 and 17 might impair the immune recognition of glioma cells via the activating immunoreceptor NKG2D by cleavage of its ligands from the cell surface. Glioblastoma-initiating cells (GIC) with stem cell properties have been identified as an attractive target for immunotherapy. However, GIC immunogenicity seems to be low. Methods and Results Here, we show that ADAM10 and ADAM17 are expressed on the cell surface of GIC and contribute to an immunosuppressive phenotype by cleavage of ULBP2. The cell surface expression of ULBP2 is enhanced upon blocking ADAM10 and ADAM17, and treatment with ADAM10 and ADAM17 specific inhibitors leads to enhanced immunorecognition of GIC by natural killer cells. Conclusions Therefore, ADAM10 and ADAM17 constitute suitable targets to boost an immune response against GIC.

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A disintegrin and metalloproteinases (ADAM) 10 and 17 modulate the immunogenicity of glioblastoma initiating cells

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None declared

Abstract

There are emerging reports that the family of a disintegrin and metalloproteinases (ADAM) are involved in the maintenance of the malignant phenotype of glioblastomas. Notably, ADAM proteases 10 and 17 might impair the immune recognition of glioma cells via the activating immunoreceptor NKG2D by cleavage of its ligands from the cell surface. Glioblastoma initiating cells with stem cell properties (GIC) have been identified as an attractive target for immunotherapy. However, GIC immunogenicity seems to be low. Here, we show that ADAM10 and ADAM17 are expressed on the cell surface of GIC and contribute to an immunosuppressive phenotype by cleavage of ULBP2. The cell surface expression of ULBP2 is enhanced upon blocking ADAM10 and ADAM17 and treatment with ADAM10 and ADAM17 specific inhibitors leads to enhanced immune recognition of GIC by natural killer cells. Therefore, ADAM10 and ADAM17 constitute suitable targets to boost an immune response against GIC.

Key words: stem cell; glioblastoma; a disintegrin and metalloproteinase (ADAM); immunotherapy; NK cell; NKG2D

Introduction

The treatment of glioblastoma remains a major challenge in the field of neurooncology. The current standard of care includes surgery, radiotherapy and alkylating chemotherapy. The median survival times with this treatment are less than 12 months according to a population-based study ¹. One promising approach to enhance the survival of glioblastoma patients is immunotherapy ^{2,3}. Glioma cells are per se prone to an attack by natural killer (NK) cells due to expression of ligands for activating immunoreceptors like NKG2D ⁴. NKG2D is a C-type lectin-like homodimeric receptor expressed by NK, $\gamma\delta$ T and CD8+ $\alpha\beta$ T cells. The ligands for *NKG2D* include the MHC class I chain-related proteins A and B (MICA/B) and UL16-binding proteins (ULBP1–6) which are not expressed by most normal tissues, but up-regulated upon malignant transformation, infection or cellular stress ^{5,6}. MICA, MICB and ULBP1-3 are expressed on the cell surface of human glioma cells ^{7,8}. In a mouse model of glioma, the growth of syngeneic intracerebral tumors was inhibited by peripheral vaccination with MICA-overexpressing irradiated tumor cells and vaccination resulted in NK and T cell activation *in vivo* indicating a possible therapeutic use of the NKG2D receptor-ligand system in glioblastoma ⁷. However, the immunosuppressive microenvironment within glioblastomas impairs the NKG2D system via downregulation of cell surface expression of MICA and ULBP2 mediated by transforming growth factor (TGF)- β and cleavage by metalloproteinases ⁸.

Among these metalloproteinases, members of the a disintegrin and metalloproteinase (ADAM) family confer malignancy in several types of cancer, e.g. breast cancer or malignant gliomas ⁹. ADAMs are involved in the activation of preforms of cytokines and growth factors or shed the extracellular domains of cell surface proteins ⁹. In the human glioma cell line U87, ADAM17, also known as tumor

necrosis factor alpha converting enzyme (TACE), contributes to the malignant phenotype of these cells including promotion of cell growth, viability, invasiveness and neo-angiogenesis *in vitro* and tumor growth *in vivo* which is in part mediated by epidermal growth factor receptor - phosphoinositide 3-kinase/AKT signaling ¹⁰. ADAM10 promotes glioma cell migration by cleavage of the adhesion molecule N-cadherin from the cell surface in a protein kinase C-dependent manner ¹¹. Moreover, ADAM10 and ADAM17 might even be involved in the maintenance of the stem cell phenotype of GIC ¹². Notably, ADAM10 and ADAM17 cleave MICA and ULBP2 from the cell surface of B cell line C1R, the embryonic fibroblast cell line 293T and cervical, mammary, prostate and pancreatic carcinoma cell lines ^{13,14}. However, to date little is known about a possible role of ADAM10 and ADAM17 in the regulation of cell surface expression of NKG2D ligands (NKG2DL) and thus a possible modulation of immunogenicity in glioma cells.

A crucial issue for an effective immunotherapy is the choice of the target. In recent years, there is growing evidence for the presence of glioma-initiating cells within glioblastomas owing stem cell properties ¹⁵. We here refer to these cells as glioma initiating cells (GIC) in the following. In a hierarchical tumor model, GIC are crucial for the initiation and maintenance of glioblastomas and therefore constitute an attractive therapeutic target. GIC are defined by the stem cell properties self-renewal, multipotency and tumorigenicity, forming tumors resembling the initial human tumors ^{16,17}. Current treatments might spare enough GIC to allow regrowth of the tumors. Despite the expression of ligands on GIC for activating immunoreceptors like NKG2D or NKp46 ^{18,19}, several immunosuppressive mechanisms of GIC have been described which might lead to immune evasion. This includes the induction of regulatory T cells or the inhibition of proliferation and the apoptosis of T cells *in vitro*,

in part mediated by signal transducer and activator of transcription 3 (STAT3)^{20,21}. A defective antigen processing machinery in GIC enhances their ability to evade a T cell-mediated immune response¹⁹. We have previously defined a contribution of the atypical human leukocyte antigen (HLA)-E to this immunosuppressive phenotype of GIC towards innate immunity²².

In the present work, we describe the modulation of immunogenicity of GIC by membrane-bound ADAM10 and ADAM17. Blocking of ADAM10 and ADAM17 with specific inhibitors or the use of small interfering RNA (siRNA) decreases cleavage from the cell surface and therefore, as a direct consequence the cell surface expression of ULBP2 is enhanced. Treatment with ADAM10 and ADAM17 specific inhibitors leads to enhanced immune recognition of GIC in cytotoxicity assays and to enhanced release of interferon (IFN)- γ by NK cells in co-culture with these GIC. Therefore, ADAM10 and ADAM17 constitute suitable targets to boost an immune response against GIC.

Materials and methods

Materials and cell lines

The human malignant glioma cell line LN-229 was originally provided by Dr N. de Tribolet (Lausanne, Switzerland) and renamed LNT-229 for clarification (T for Tübingen). The cells were maintained in DMEM supplemented with 2 mM L glutamine and 10% fetal calf serum (FCS) (all Invitrogen, Basel, Switzerland). The GIC lines GS-2, GS-5, GS-7 and GS-9 have been characterized for stemness properties earlier ²³. In summary, the cell lines expressed the stemness markers Sox2 and nestin as undifferentiated cultures and either neuronal (MAP2, neurofilament), oligodendroglial (galactocerebroside C) or glial markers (glial fibrillary acidic protein) upon differentiation. Moreover, the GIC lines were characterized by self-renewal and their tumorigenicity was confirmed *in vivo*. All GIC lines were cultured in 75 cm² culture flasks and maintained in neurobasal medium with B-27 supplement (20 µl/ml) and glutamax (10 µl/ml) from (all Invitrogen) fibroblast growth factor (FGF)-2, epidermal growth factor (EGF) (20 ng/ml each; Peprotech, Rocky Hill, PA) and heparin (32 IE/ml; Ratiopharm, Ulm, Germany). Stem cell factors were supplemented twice a week, complete medium changed once a week. Cells were passaged when spheres reached an estimated diameter of 500 µm or an estimated density of 5×10^4 cells/cm². Spheres were dissociated mechanically and enzymatically. Briefly, we spun down the cells and resuspended the pellet in 1 ml accutase (PAA, Wien, Austria). After mechanical dissection by pipetting up and down, we incubated the cells at 37°C for 5 minutes. From previous work we know that accutase does not alter the expression level of NKG2DL on the cell surface of glioma cells ^{4,8}. The NK cell line NKL ²⁴ was cultured in RPMI 1640 medium (PAA)

containing 15% fetal calf serum (FCS), 2 mM L-glutamine (Gibco Life Technologies, Paisley, UK), penicillin (100 IU/ml)/streptomycin (100 mg/ml) (Gibco), 1 mM sodium pyruvate and 100 U/ml interleukin 2 (Peprotech). Specific inhibitors for ADAM10 (GI254023X) and ADAM10 and 17 (GW280264X) were from Glaxo Smith Kline (London, UK). GI254023X is considered being 100 fold more specific in blocking ADAM10 than ADAM17 ²⁵. SD-208 ²⁶, a transforming growth factor (TGF)- β receptor I kinase inhibitor specially targeting activin receptor-like kinase 5, was purchased from Tocris Bioscience (Bristol, UK).

Monoclonal antibodies and flow cytometry

Cell surface expression was assessed with the following monoclonal antibodies (mAbs): ADAM10 (Clone 163003, APC-conjugated; mouse IgG2b; R&D Systems, Minneapolis, MA) and ADAM17 (Clone 111633, Fluorescein-conjugated; mouse IgG1; R&D Systems) and hybridoma supernatants: MICA (clone AMO1, unconjugated, mouse IgG1), MICB (clone BMO1, unconjugated, mouse IgG1), ULBP1-3 (clones AUMO1; BUMO1 and CUMO3, all unconjugated, mouse IgG1 ²⁷). Isotype matched antibodies unconjugated IgG1 (clone MOPC 21; Sigma-Aldrich, Buchs, Switzerland), FITC-conjugated IgG1 (clone MOPC 21; Sigma-Aldrich) and APC-conjugated IgG2b (clone MPC-11; Biolegend, San Diego, CA) were used as controls. The PE-conjugated goat anti-mouse IgG (Dako, Freiburg, Germany, dilution 1:20) was used as secondary antibody where appropriate. The cells were dissociated as described, preincubated in PBS with 2% FCS and stained with specific mAb or matched mouse Ig isotype for 30 min on ice, followed by incubation with the PE-conjugated secondary antibody for 30 min where appropriate. Then the

cells were washed with PBS containing 2% FCS. Flow cytometry was performed with a CyAn ADP Analyzer (Beckman Coulter, Nyon, Switzerland). Specific fluorescence index (SFI) was calculated by dividing median fluorescence obtained with the specific antibody by median fluorescence obtained with the control antibody.

Viability and proliferation

2.5×10^5 GIC were seeded in a 24 well plate in the presence of increasing concentrations of GI254023X, GW280264X (both 3 μ M) or DMSO for 48 h. Viability and cell concentration were assessed by Trypan blue staining using Vi-CELL™ XR (Beckman Coulter, Nyon, Switzerland). The cell count was calculated based on the cell concentration and the predetermined cell culture volume.

Limiting dilution sphere formation assay

For the sphere formation assays, GS-5 cells were mechanically and enzymatically dissociated to a single cell suspension with accutase, diluted and seeded in triplicates at 10, 100, 1000 and 10.000 cells/well in a 96 well plate under stem cell culture conditions. After 24 h, 50 μ l stem cell medium containing GI254023X, GW280264X (both to a final concentration of 3 μ M) or DMSO were added. After 7 days, another 100 μ l of stem cell medium containing inhibitors (final concentration 3 μ M) or DMSO was supplemented. The number of spheres was counted in each well after 14 days.

Immunocytochemistry

For undifferentiated GIC, cytopsin samples were prepared. For differentiation, GIC were dissociated and dissolved in neurobasal A medium containing B-27 supplement (20 μ l/ml), FCS (10%), and glutamax (10 μ l/ml) (all Invitrogen) as well as cyclic adenosine monophosphate (cAMP) (0.75 mM) and retinoic acid (1 mM) (both Sigma-Aldrich)²³. GIC were seeded on glass coverslips in a 24 well plate (both TPP, Trasadingen, Switzerland) for 8 days. Cytopsin samples and coverslips were either fixed in methanol for glial fibrillary acidic protein (GFAP) or 4% paraformaldehyde for nestin, β -3-tubulin and galactocerebroside C (GalC), treated with H₂O₂ and blocked with PBS containing 10% swine serum and 0.3% Triton X. The following antibodies were used for primary stem cell staining: nestin (clone 10C2, IgG1; Zytomed, Berlin, Germany; diluted 1:100), GFAP (ab Z0334, polyclonal rabbit IgG; Dako; diluted 1:1000), β -3-tubulin (ab18207, polyclonal rabbit IgG; Abcam, Cambridge MA; diluted 1:1000) and GalC (MAB342, mouse IgG3, Millipore, Billerica, MA; diluted 1:200). As isotype controls, we used mouse IgG1 (Dianova, Hamburg, Germany), mouse IgG3 (eBioscience, Vienna, Austria) or polyclonal rabbit IgG (Vector Laboratories, Burlingame, CA). The cells were incubated at 4°C overnight. Goat anti-mouse IgG Alexa Fluor 594-coupled secondary antibody (Invitrogen, A11032) or goat anti-rabbit IgG Alexa Fluor 594-coupled secondary antibody (Invitrogen, A11034) were used at 1:100. 4',6-Diamidino-2-Phenylindole (DAPI) for nuclear counterstain was from Invitrogen (D3571) and used at 1:2000. Slides were mounted using fluorescence mounting medium (Dako; S3023). Images were acquired by using an Axioplan II fluorescence microscope (Carl Zeiss AG, Feldbach, Germany).

Real-Time PCR

RNA was prepared from the cell lines using NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany). Subsequently, cDNA was generated using SuperScript II Reverse Transcriptase (Invitrogen). RNA and DNA concentrations were determined using NanoDrop (Thermo Scientific, Waltham, MA).

cDNA amplification was monitored using SYBRGreen chemistry on the ABI PRISM7000 Sequence Detection System (Applied Bio-Systems, Weiterstadt, Germany) using the following specific primers (forward/reverse): *GAPDH*: described in ²². *ADAM10*: 5'-GGATGGAAAAGGAACCCAAT-3', 5'-ACGAAGAACAGGGAACATGG-3'; *ADAM17* 5'-TTGAGCGATTTTGGGATTTC-3', 5'-AAACCAGAACAGACCCAACG-3'. Primers for *MICA*, *MICB* and *ULBP1-3* have been published ²⁸. Data analysis was done using the DDCT method for relative quantification. Briefly, threshold cycles (CT) for *GAPDH* RNA (reference) and specific primers (sample) were determined in duplicate. The expression levels were determined following the formula: $rl=2^{-(C_{T_Target} - C_{T_GAPDH})}$ and then referred to whole normal brain cDNA obtained by Lonza (Walkersville, MD).

RNA interference

To silence *ADAM10* and *ADAM17* expression, the glioma cells were transfected with *ADAM10* and *ADAM17* ON-TARGETplus SMART Pool siRNA (Dharmacon, Lafayette, CO) or non-targeting SMART pool siRNA (Dharmacon) as a control respectively. Glioma cells were seeded in 6 well plates overnight and subsequently

transfected with siRNA using lipofectamine RNAi (Invitrogen). Gene silencing was verified by real-time PCR and by flow cytometry 72 h after transfection.

Enzyme-linked immunosorbent assay (ELISA)

For the detection of soluble ULBP2 (sULBP2) in cell culture supernatants, 5×10^5 cells were cultured in 500 μ l B-27 supplement free-medium and treated as indicated. The supernatants were collected after 48 h and then analyzed by sandwich ELISA for sULBP2 using the DuoSet ELISA Kit (DuoSet DY1298; R&D Systems, Minneapolis, MA).

To assess IFN- γ secretion by NK, 2×10^4 GIC were pretreated with either GW or DMSO as a control for 24 h and subsequently co-cultured with 4×10^5 NK cells in 500 μ l serum free RPMI medium for 12 h. The conditioned media from this co-culture were collected and analyzed using the IFN- γ ELISA MAX standard kit obtained from Biologend (Uithoorn, Netherlands).

Cytotoxicity assay

We used a flow cytometry-based cytotoxicity assay²⁹ which employs the membrane dye PKH-26 and the viability dye ToPro3 as described²². Briefly, the target cells were incubated with PKH-26 dissolved in Diluent C (1:250; all from Sigma–Aldrich) for 3 min. Staining was stopped by washing with RPMI 1640 medium containing 10% FCS. Then the effector and target cells were coincubated at varying effector to target (E:T) ratios for 3 h. ToPro3 (Invitrogen) was added to the vials prior to measurement and incubated for 30 seconds. The percentage of target cell lysis was determined by

flow cytometry using the CyAn ADP Analyzer (Beckman Coulter). Background elimination was done by subtraction of spontaneous lysis of target cells only. All experiments were done in duplicates.

Statistical analysis

The experiments were performed 2 to 3 times as indicated. Statistical significance was calculated by student's two-tailed t-test (* $p < 0.05$; ** $p < 0.01$) or by ANOVA, using GraphPad Prism software, version 5.0 (La Jolla, California) where indicated.

Results

GIC lines express NKG2DL

The expression of *NKG2D* ligands in the glioma cell line LNT-229 and in GIC lines GS-2, GS-5, GS-7 and GS-9 was assessed by real-time PCR and flow cytometry (Figure 1). All investigated cell lines variably express NKG2DL on the cell surface, predominantly MICA and ULBP2 (Figure 1B, C). This was only roughly paralleled by the expression on mRNA level (Figure 1A), indicating additional mechanisms of posttranscriptional and/or –translational regulation as they are well described for regulation of NKG2DL cell surface expression³⁰.

GIC lines express *ADAM10* and *ADAM17* on the cell surface

The expression of ADAM10 and ADAM17 in GIC was assessed by real-time PCR and flow cytometry (Figure 2). *ADAM10* and *ADAM17* genes are transcribed in all of the investigated GIC lines with expression levels being higher in GS-5, GS-7 and GS-9 compared to GS-2 (Figure 2A). In parallel, ADAM10 and, to a lesser extent, also ADAM17 protein can also be detected on the cell surface of GIC (Figure 2B, C). In line with the relative low mRNA expression levels of *ADAM10* and *ADAM17* in GS-2, cell surface protein expression for ADAM10 was lower than in the other GS lines and only marginal for ADAM17 (Figure 2B, C). While exhibiting strong expression of ADAM10, the cell surface level of ADAM17 in GS-9 was low (Figure 2B, C). GS-7 showed the strongest cell surface expression level of ADAM17 of the GIC lines tested here and in addition also high levels of ADAM10.

The inhibition of ADAM10 and ADAM17 leads to an upregulation of ULBP2 cell surface expression on GIC

We next assessed the role of ADAM10 and ADAM17 in the regulation of NKG2DL expression. GS-2, GS-5, GS-7 and GS-9 cells were treated with specific inhibitors GI254023X (for ADAM10) and GW280264X (for ADAM10 and ADAM17). The level of NKG2DL was assessed by flow cytometry. We found a significant upregulation of ULBP2 in all investigated cell lines (Figure 3A) in a time- and concentration-dependent manner (shown exemplarily for GS-7, Figure 3B). While the expression levels of MICB, ULBP1 and ULBP3 were unaltered, we observed a trend towards upregulation of MICA in GS-5 (Figure 3A). Next, we assessed the presence of sULBP2 in conditioned cell culture supernatants of LNT-229 and GIC cultures by ELISA. sULBP2 concentrations in the supernatants decreased significantly following treatment with GI254023X and were below the detection limit upon treatment with GW280264X, while the level of *ULBP2* on the mRNA level remained largely unaltered as assessed by real-time PCR (Figure 3C, D). In order to confirm the specificity of the effects mediated by the inhibitors, we used small interfering RNA to transiently silence *ADAM10* and *ADAM17* gene expression in GS-7. We achieved an efficacy of gene silencing of more than 90% as assessed by real-time PCR (Figure 4A). The knock-down of ADAM10 and ADAM17 cell surface expression was verified by flow cytometry (Figure 4B). The knock-down of either *ADAM10* or *ADAM17* gene expression as well as the combined knock-down led to a significant upregulation of ULBP2 on the cell surface while the expression of the other NKG2DL was largely unaffected (Figure 4D, E) similar to the effect mediated by the inhibitors

We assessed the proliferation and survival of GIC upon treatment with the ADAM inhibitors or knock-down of ADAM expression. The viability of GIC was not altered

following treatment with the inhibitors or upon knock-down. This is shown exemplarily for the inhibitors in GS-7 (Suppl. Figure 1A). The proliferation of GS-7 cells was significantly reduced upon treatment with GW280264X or ADAM10/17 co-knockdown (Suppl. Figure 1B and data not shown) respectively, consistent with previous data¹².

Blocking of TGF- β signaling does not alter upregulation of ULBP2 cell surface levels mediated by inhibition of ADAM10 and ADAM17

In order to investigate a possible involvement of endogenous TGF- β in the ADAM10 and ADAM17-mediated effects on ULBP2 cell surface expression we used SD-208, a TGF-beta receptor I kinase inhibitor, which efficiently blocks TGF- β signaling in glioma cells²⁶. GS-7 cells were treated with SD-208 for 48 h and blockade of SMAD2 phosphorylation was verified by immunoblot (data not shown). The expression of ADAM10 and ADAM17 remained unaltered as assessed by flow cytometry (Figure 5A). We observed a tendency to enhanced expression of MICA and ULBP2, though statistically not significant (Figure 5B). Next, the cells were treated with GW280264X for 24 h and no further increase in MICA expression was observed. However, expression of ULBP2 in SD-208 treated cells was markedly increased (Figure 5B), suggesting that cleavage of ULBP2 by ADAM10 and ADAM17 is largely independent of TGF- β .

The inhibition of ADAM10 and ADAM17 enhances immunogenicity of GIC

To define a role for ADAM10 and ADAM17 in modulating the immunogenicity of GIC, we used either GI254023X or GW280264X pretreated GS-7 or GS-9 cells as targets in a cytotoxicity assay with NKL cells as effectors. We observed a significantly increased susceptibility towards NK cell mediated lysis following treatment with the ADAM10 and ADAM17 specific inhibitor GW280264X and to a lesser extent by the ADAM10 specific inhibitor GI254023X in GS-7 (Figure 6A). In GS-9, treatment with ADAM inhibitors led to enhanced susceptibility towards NK cells, however the effect of GW280264X was not superior to GI254023X (Figure 6B). The relative increase of ULBP2 expression following treatment with GW280264X compared to GI254023X was highly significant in GS-7 cells ($p=0.008$) and present to a lesser degree in GS-9 ($p=0.02$) (Figure 3A). Thus, the slight additional upregulation of ULBP2 levels in GS-9 by GW280264X compared to GI254023X might not be sufficient to be translated in an enhanced immune recognition by NKL cells. Since the secretion of IFN- γ by activated NK cells is considered an important link between the innate and adaptive immune response, we analyzed the conditioned media of NKL and GS-7 co-cultures for the level of IFN- γ by ELISA. NKL cells were co-cultured with GS-7 cells pre-treated or not with GW280264X (3 μ M for 48 h). The levels of IFN- γ in the supernatant of co-cultures with GS-7 cells pre-treated with GW280264X were significantly increased (Figure 6C).

Discussion

In this study, we define a role of ADAM10 and ADAM17 for the modulation of immunogenicity of GIC by influencing the NKG2D receptor-ligand system. The NKG2DL MICA, MICB and ULBP1-3 are expressed on the cell surface of GIC lines. The expression of NKG2DL in gliomas, especially of MICA and ULBP2, is known to be influenced by various factors, including downregulation by TGF- β and cleavage by metalloproteinases ⁸. ADAM10 and ADAM17 belong to a family of proteases involved in the shedding of extracellular domains of cell surface proteins ⁹. Both proteins cleave NKG2DL, namely MICA and ULBP2 as demonstrated in malignant and non-malignant cell lines ^{14,31}. A single report provides evidence that ADAM17 is crucially involved in the shedding of soluble MICB from the cell surface of U373 transfectants overexpressing MICB within detergent-resistant membrane microdomains (DRM) ³². In this work, we investigated the role of ADAM10 and ADAM17 in modulating the cell surface expression of NKG2DL in GIC. These GIC lines have been described before for their stemness properties ²³. We adhered to the stem cell permissive culture conditions to assure maintenance of the stem cell phenotype and verified self-renewal capacity and multipotency of GIC. ADAM10 is expressed on all GIC lines investigated here. The strongest ADAM17 expression was present in GS-7. Interference with ADAM10 and ADAM17 function by specific pharmacological inhibitors or gene silencing with siRNA led to a strong upregulation of ULBP2 expression on the cell surface. sULBP2 concentrations in the supernatants decreased significantly following treatment with the inhibitors, while the level of *ULBP2* on the mRNA level remained unaltered, underscoring the view that ADAM10 and ADAM17 decrease ULBP2 cell surface expression via proteolytic cleavage.

Few reports indicate a link between effects mediated by ADAM proteases and TGF- β . TGF- β -mediated effects on glioma cell motility and invasiveness could be abrogated in part by blocking ADAM17³³ and ADAM12 expression was upregulated by TGF- β in mammary carcinoma cells³⁴. The cleavage of ULBP2 from the cell surface by ADAM10 and ADAM17, however, seems to be largely independent from TGF- β . Previously, the abrogation of TGF- β signaling led to an upregulation of MICA and ULBP2 on non-GIC⁸. We observed a tendency to enhanced expression of MICA and ULBP2 on the cell surface of GS-7 cells treated with SD-208, though statistically not significant. These cells were subsequently treated with GW280264X for 24 h and no further increase in MICA expression was observed. However, the levels of ULBP2 in these cells with blocked TGF- β signaling were markedly increased, showing that increased ULBP2 levels upon inhibition of ADAM10 and ADAM17 are largely independent from TGF- β , but rather due to a direct blockade of ADAM-mediated ULBP2 cleavage.

In contrast to previous observations in HeLa cervix carcinoma cells¹⁴, we found a minor upregulation of MICA only in GS-5 following impairment of ADAM10 and ADAM17. The sheddase activity of ADAM10 and ADAM17 is not dependent on a specific sequence motif but rather on the length of the stalk region of the NKG2DL¹⁴. Notably, the most common MICA variant MICA08 is not cleaved by ADAM proteases, but released in endosomes³⁵. This might explain variations in NKG2DL cleavage observed in different cell lines of different tissue origin^{14 32} and our findings. Moreover, shedding of MICA seems to depend critically on its association to endoplasmic reticulum protein 5 (ERp5)³⁶. An impairment of this association in our GIC lines might be involved in the lack of shedding of MICA, a possible mechanism which warrants further investigation. The inhibitors used here are highly specific for

ADAM10 (GI254023X) or ADAM10 and ADAM17 (GW280264X) respectively. The specificity of GI254023X and GW280264X has been repeatedly confirmed and these inhibitors have been used in various subsequent investigations^{12-14,25,37}. Moreover, we used RNA interference to silence *ADAM10* and *ADAM17* expression in a sequence-specific manner using appropriate controls and obtained the same results as with the pharmacological inhibitors.

Collectively, our data provide strong evidence that, in accord with the present literature, ULBP2 expression on GIC is substantially reduced through cleavage by ADAM10 and ADAM17. We do not assume that the cleavage of NKG2DL is a specific mechanism in GIC since the sheddase activity of ADAM10 and ADAM17 depends on the length of the stalk region of the NKG2DL as described above. However, it is of greater importance that, when targeting ADAM proteases to enhance immune response against glioma cells, the GIC are not spared.

To date, little is known about the role of ADAM proteases in regulating immune function in glioma. We show here, that inhibition of ADAM10 and ADAM17 leads to enhanced lysis of GIC by NK cells. Moreover, we found significantly increased levels of IFN- γ in the supernatant of co-cultures of NK cells with GIC pre-treated with an ADAM inhibitor. This is in line with the results from a recent report investigating other tumor entities than glioma¹³. Since IFN- γ is a main regulator of T-cell activity, inhibition of ADAM10 and ADAM17 and subsequent upregulation of ULBP2 might therefore also enhance adaptive immune responses through induction of IFN- γ secretion by NK cells.

As outlined earlier, ADAM10 and ADAM17 are furthermore involved in the maintenance of the malignant phenotype of glioma cells regarding migration,

invasion, neo-angiogenesis or proliferation ^{10,11}. Recently, a role for ADAM10 and ADAM17 for the maintenance of sphere formation and self-renewal of sphere forming cells derived from human gliomas kept under stem cell culture conditions was proposed ¹². Here we focused on the modulation of the immunophenotype of GIC by ADAM proteases rather than the stem cell phenotype. Nevertheless, we reproduced the finding of reduced proliferation and decreased number of spheres in a limiting dilution assay upon inhibition of ADAM10 and ADAM17 (Suppl. Figure 3) as previously reported ¹².

In summary, we demonstrate that ADAM10 and ADAM17 are involved in the modulation of immunogenicity of glioblastoma stem-like cells. ADAM10 and ADAM17 cleave ULBP2 from the cell surface and therefore warrant further investigation as possible targets to boost an immune response towards these cells.

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Figure legends

Fig. 1: GIC lines express NKG2D ligands. The expression of NKG2DL in GIC was assessed on the transcriptional and on the cell membrane level. (A) *MICA*, *MICB* and *ULBP1-3* mRNA expression was analyzed by real-time PCR. Data are expressed relative to normal brain cDNA and represent mean relative expression \pm SD from 3 independent experiments. (B, C) GIC were stained with mAbs for MICA (clone AMO1), MICB (clone BMO1), ULBP1 (clone AUMO1), ULBP2 (clone BUMO1), ULBP3 (clone CUMO3) or isotype-matched Ig, followed by staining with a PE-labelled anti-mouse secondary antibody. Flow cytometry was performed and data in (B) represent mean SFI values \pm SD from 3 independent experiments (SFI=1 is marked with dotted line); ** $p < 0.01$, * $p < 0.05$; student's two tailed t-test). In (C), representative histograms from flow cytometry including the respective SFI values are shown (curve in light grey: isotype control, dark grey: specific MICA,B or ULBP1-3 mAb).

Fig. 2: GIC lines express ADAM10 and ADAM17 on the cell surface. (A) *ADAM10* and *ADAM17* mRNA expression was determined by real-time PCR. Data are expressed relative to the expression of normal brain cDNA and represent mean relative expression \pm SD from 3 independent experiments. (B) GIC were stained with

directly labelled mAbs for either ADAM10 (Clone 163003), ADAM17 (Clone 111633) or isotype-matched Ig. Data represent mean SFI values \pm SD from 3 independent experiments (SFI=1 is marked with dotted line). In (C), representative histograms from flow cytometry including the respective SFI values are shown (curve in light grey: isotype control, dark grey: specific ADAM10 or ADAM17 mAb).

Fig. 3: Inhibition of ADAM10 and ADAM17 using specific inhibitors leads to an upregulation of ULBP2 cell surface levels and to decrease of sULBP2 in cell culture supernatants. (A) The GIC lines GS-2, GS-5, GS-7 and GS-9 were incubated with GI254023X (for ADAM10) or GW280264X (for ADAM10 and ADAM17) respectively (3 μ M; 48 h). The cells were subsequently stained with mAbs for MICA, MICB, ULBP1-3 or isotype-matched Ig, followed by staining with a PE-labelled anti-mouse secondary antibody. Flow cytometry data represent mean SFI \pm SD from 3 independent experiments (SFI=1 is marked with a dotted line; ** $p < 0.01$, * $p < 0.05$; student's two tailed t-test). (B) GS-7 cells were treated with increasing concentrations of GI254023X or GW280264X for various time points. The cells were subsequently stained with mAb for ULBP2 or isotype-matched Ig, followed by staining with a PE-labelled anti-mouse secondary antibody. Flow cytometry data represent mean SFI \pm SD from 3 independent experiments. (C) GS-7 cells were treated with GW280264X (3 μ M; 48 h). *ULBP2* mRNA expression was determined by

real-time PCR. Data are expressed relative to the expression of normal brain cDNA and represent mean relative expression \pm SD from 3 independent experiments. (D) GIC or LNT-229 cells were treated with DMSO, GI254023X (3 μ M) or GW280264X (3 μ M) for 48 h, then cell culture supernatants were harvested. The levels of sULBP2 were assessed by ELISA. Data represent mean concentrations \pm SD from 2 independent experiments.

Fig. 4: *ADAM10* and *ADAM17* gene silencing leads to an upregulation of ULBP2 cell surface expression. The GIC line GS-7 was transfected with control (siCtrl) or siRNA targeting *ADAM10* (siADAM10) or *ADAM17* (siADAM17). (A,B) The cells were harvested 72 h later and knock-down efficiency was confirmed by real-time PCR (A). Data are expressed as percentage of *ADAM10* and *17* expression in cells following *ADAM10/ADAM17* gene silencing relative to control siRNA and represent mean relative expression \pm SD from 3 independent experiments. (B) The knock-down efficiency was further confirmed by flow cytometry with ADAM10 or ADAM17 specific mAB (curve in light grey: isotype control, dark grey: specific ADAM10 or ADAM17 mAb). (C, D) GS-7 cells transfected with either siADAM10, siADAM17 or both were stained with mAbs for MICA, MICB, ULBP1-3 or isotype-matched Ig, followed by staining with a PE-labelled anti-mouse secondary antibody.

Flow cytometry data represent mean SFI values \pm SD from 3 independent experiments (** $p < 0.01$, * $p < 0.05$; student's two tailed t-test).

Fig. 5: Blocking of TGF- β signaling does not alter upregulation of ULBP2 cell surface levels mediated by inhibition of ADAM10 and ADAM17. (A) GS-7 cells were treated with SD-208 (2 μ M) or DMSO for 48 h. The levels of ADAM10 and ADAM17 at the cell surface were assessed by flow cytometry. In (B) the cells were treated as in (A) followed by treatment with either GW280264X (3 μ M, 24 h) or DMSO. The levels of MICA, MICB and ULBP1-3 were assessed by flow cytometry. Flow cytometry data represent mean SFI values \pm SD from 3 independent experiments.

Fig. 6: The inhibition of ADAM10 and ADAM17 enhances immunogenicity of GIC. (A) GS-7 and (B) GS-9 cells were incubated with GI254023X or GW280264X (3 μ M; 48 h) and then used as targets in a cytotoxicity assay with NKL cells as effectors. Target cells were stained with the membrane dye PKH-26 and then co-cultured with NKL cells at various effector to target (E:T) ratios for 3 h in duplicates. The cells were stained with the viability marker ToPro3 to identify the lysed cells by flow cytometry. Specific lysis was calculated by subtraction of background lysis

from the fraction of dead cells (n=2). Statistics were done by ANOVA (** p=0.01*, p=0.05 for GI254023X pretreated, + p=0.05 for GW280264X pretreated cells.) (C) GS-7 cells were pretreated with either GW280264X (3 μ M, 48 h) or DMSO, and then co-cultured with NKL cells (12 h, effector to target ratio 20:1). Conditioned media were collected from co-cultures or NKL cells alone and concentration of IFN- γ was assessed by ELISA. Results represent mean concentrations \pm SD of two independent experiments.

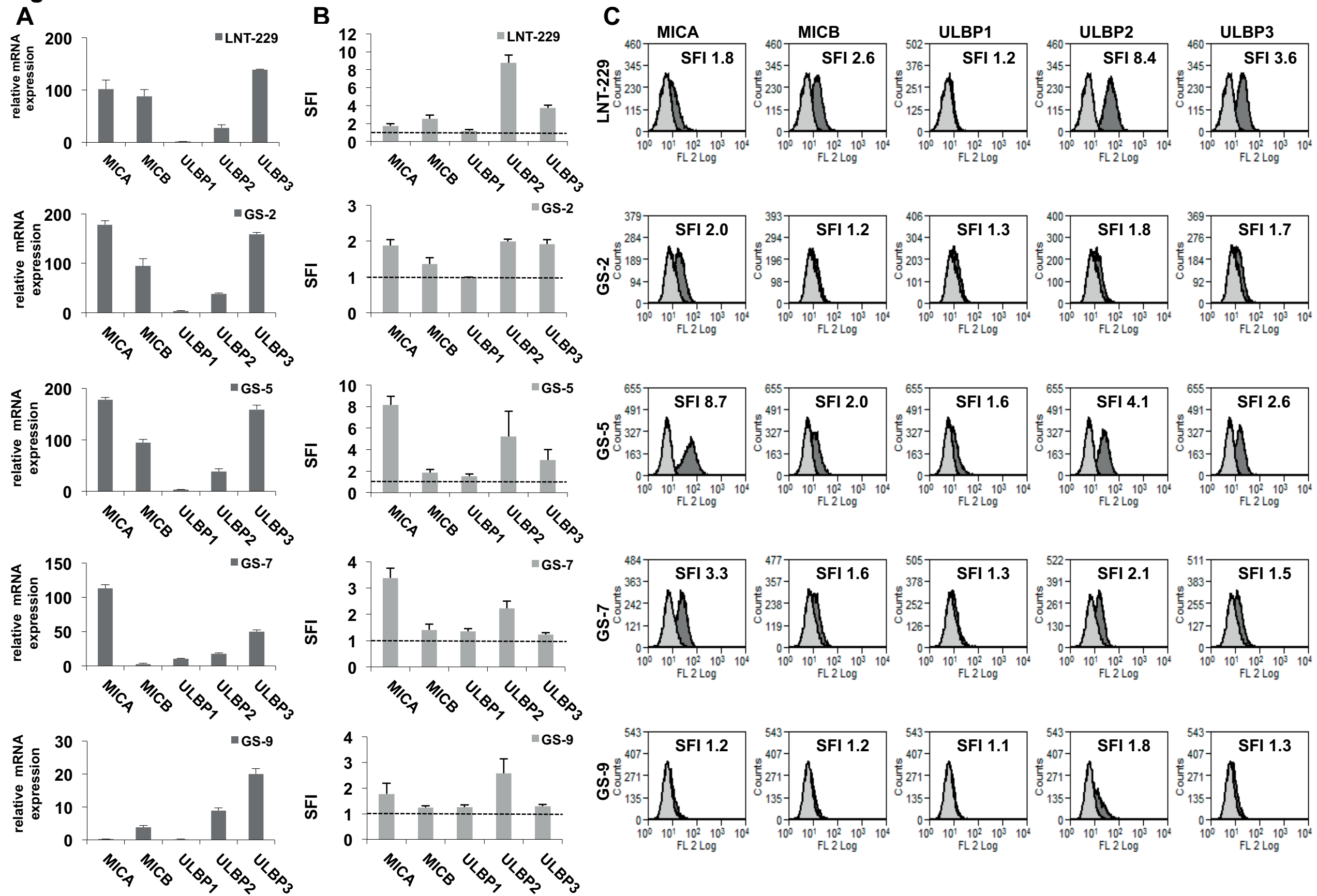
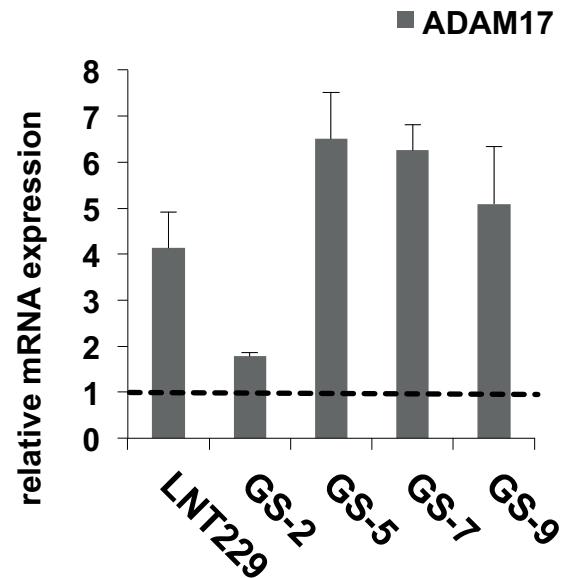
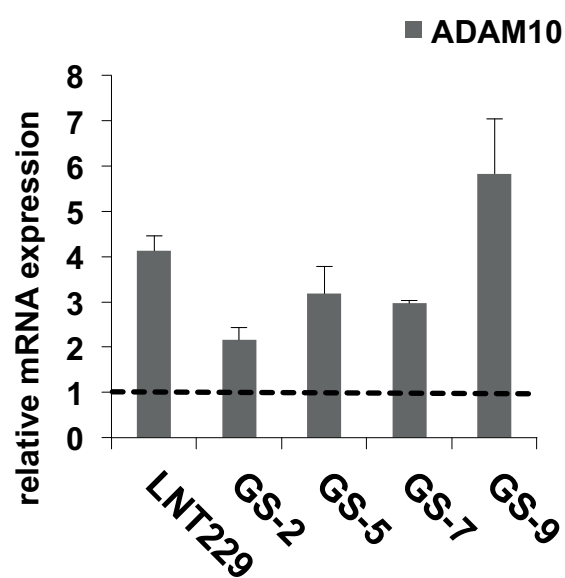
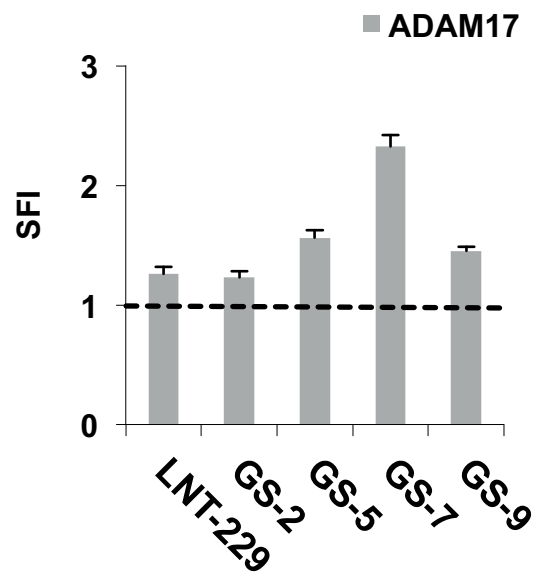
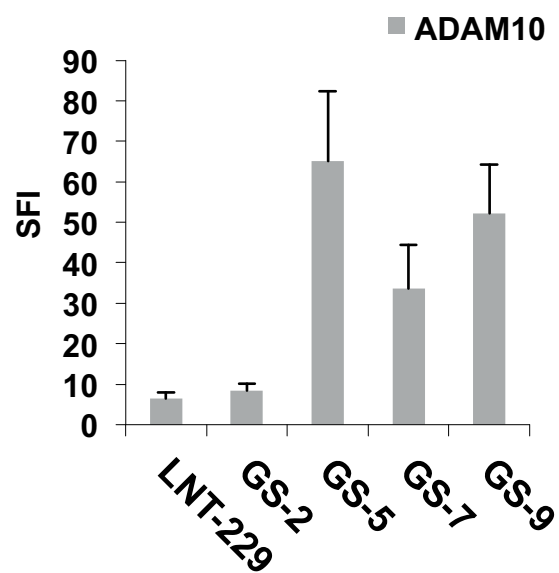
Figure 1

Figure 2

A



B



C

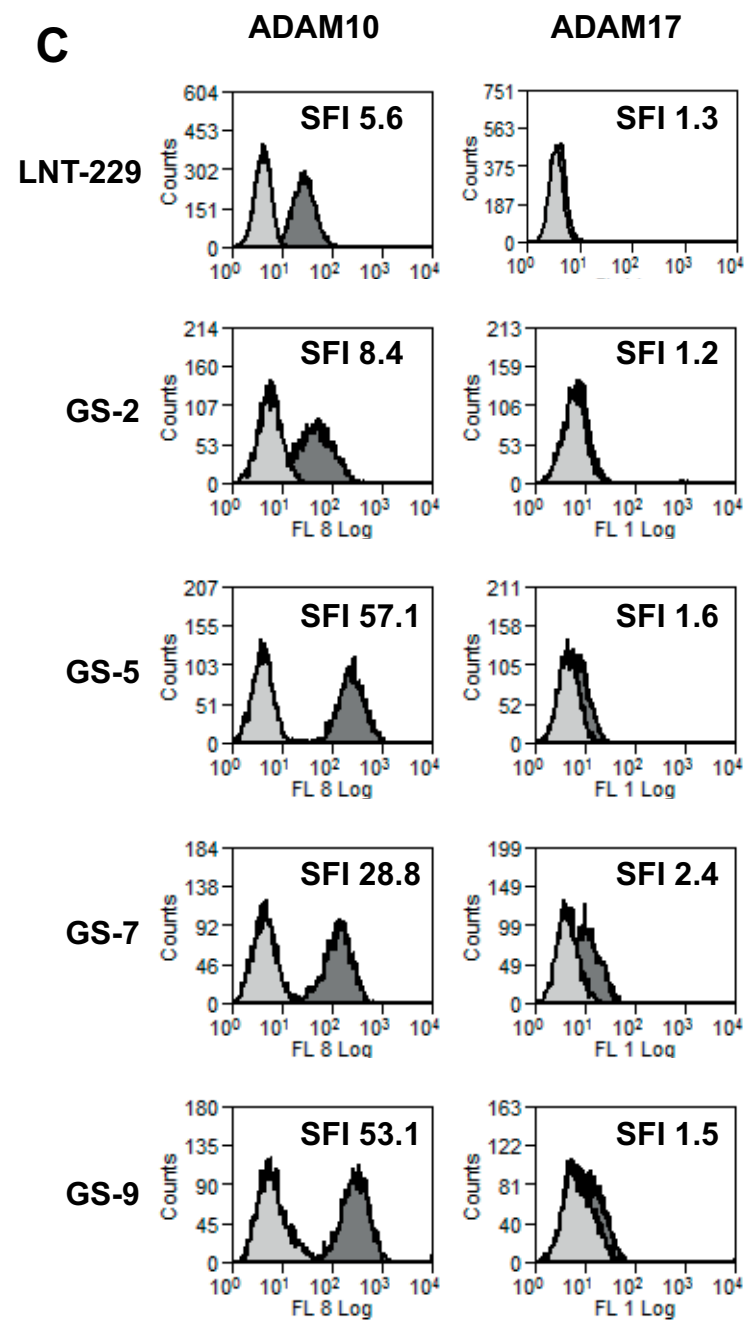


Figure 3

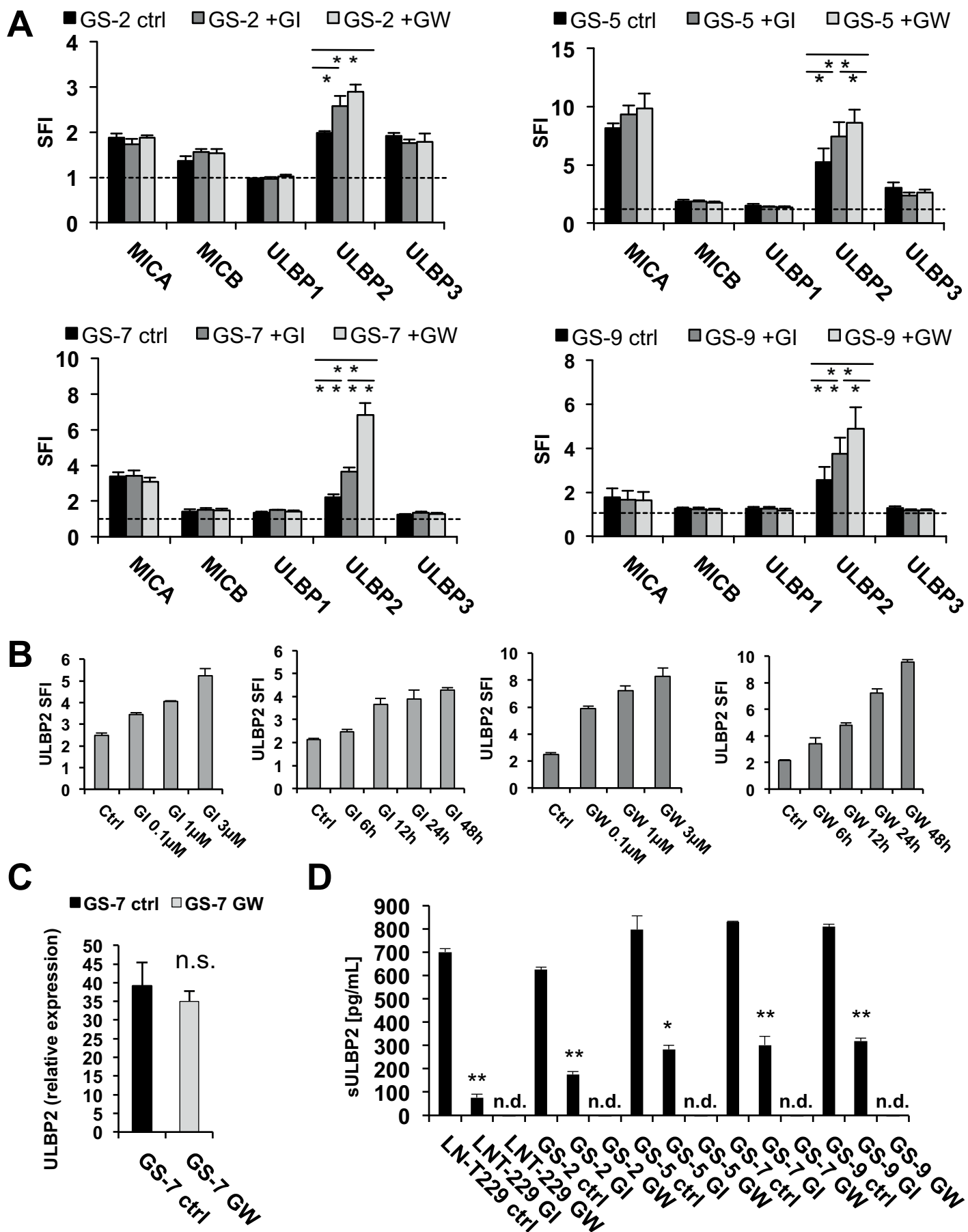


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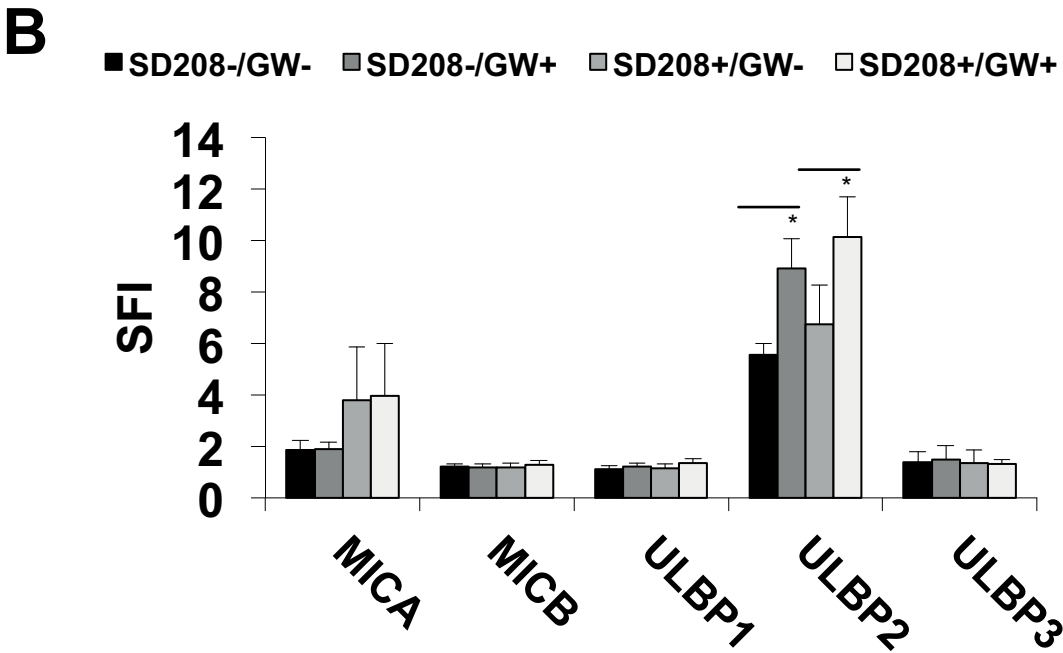
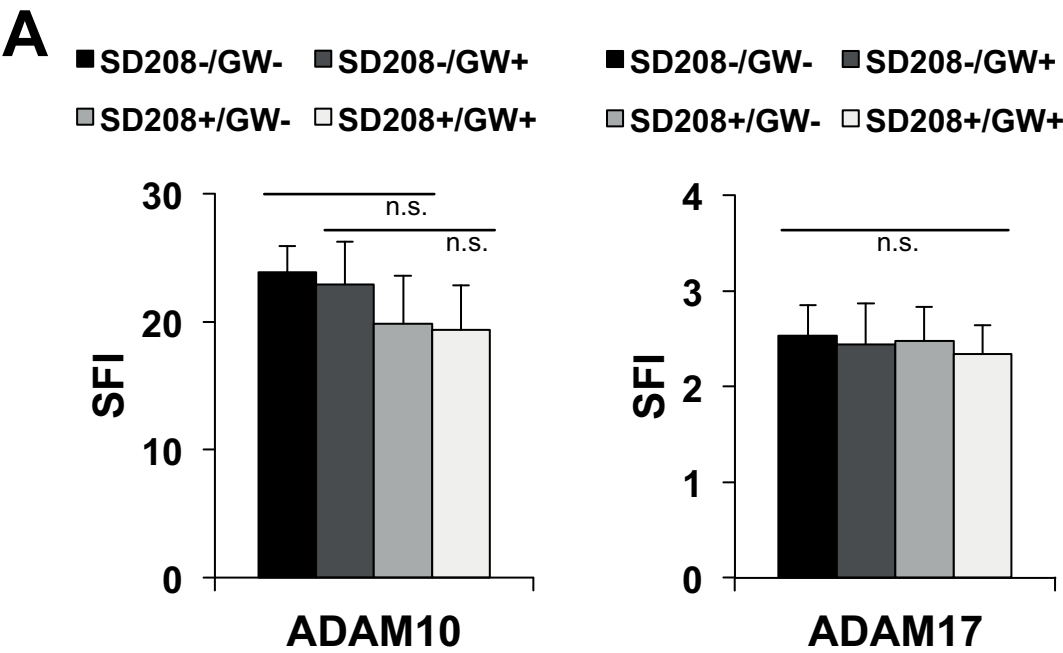
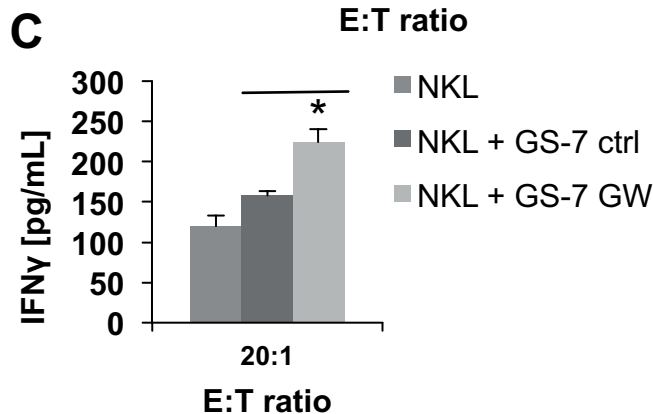
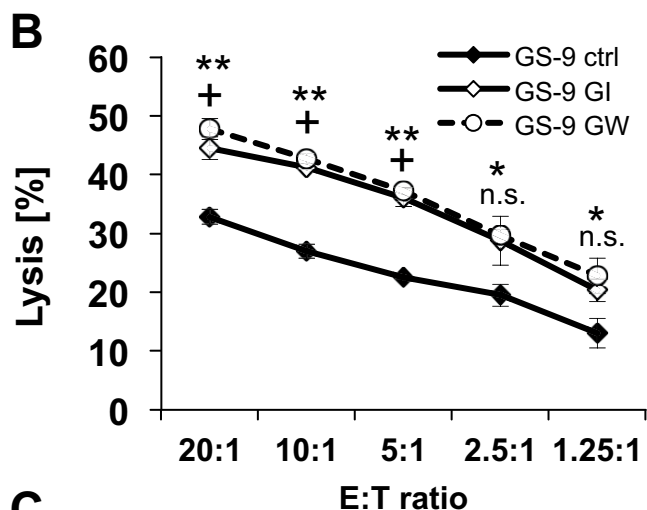
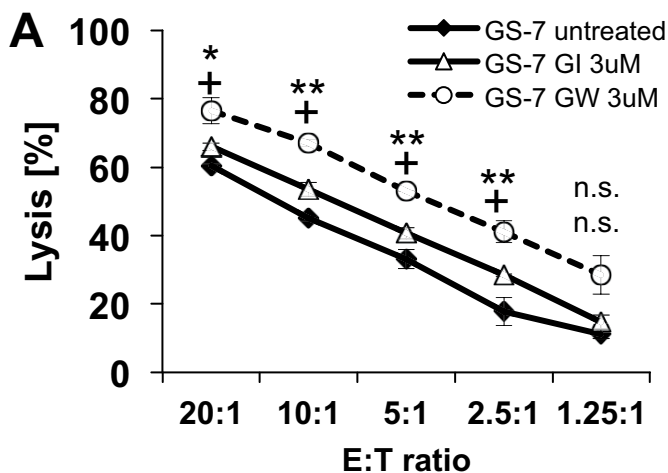
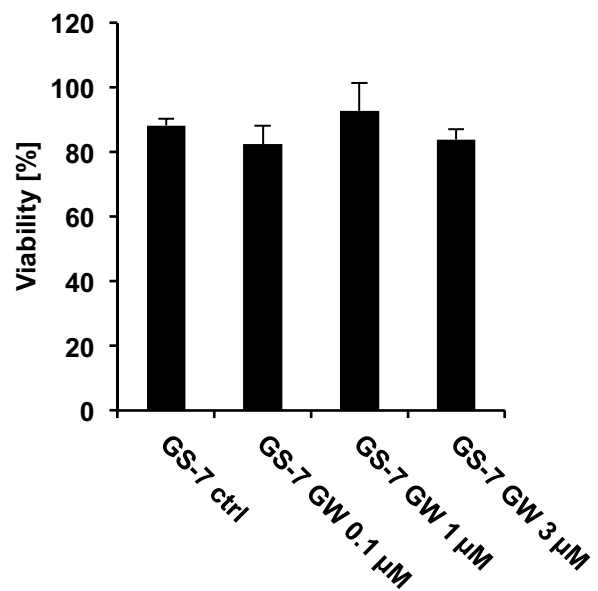
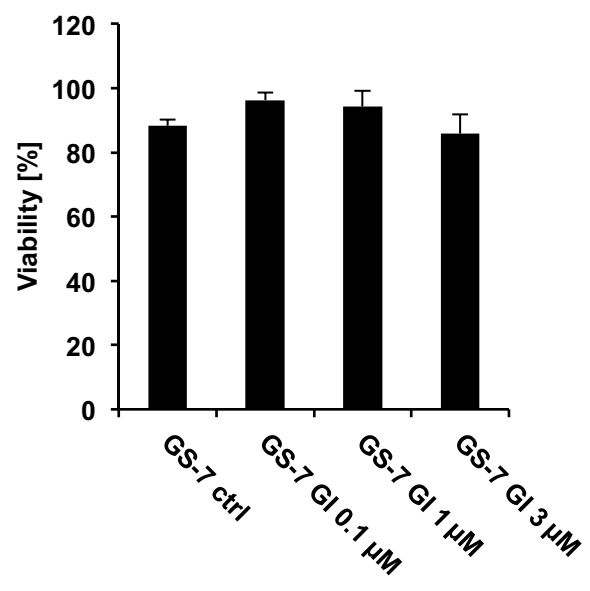


Figure 6

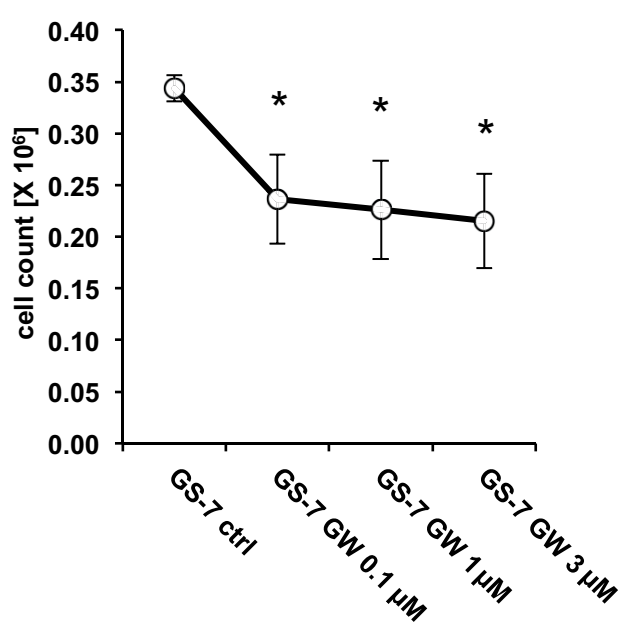
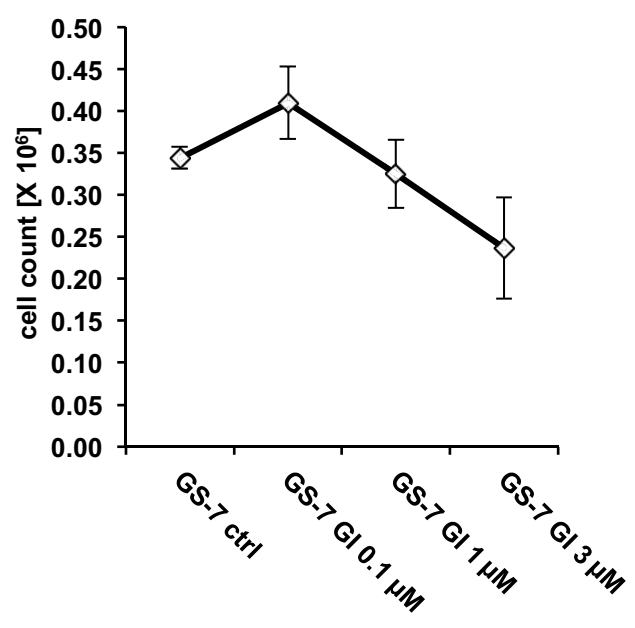


Supplementary Figure 1

A

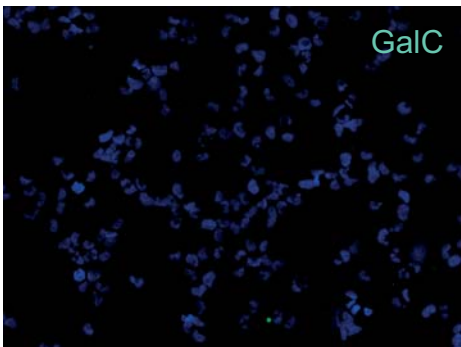
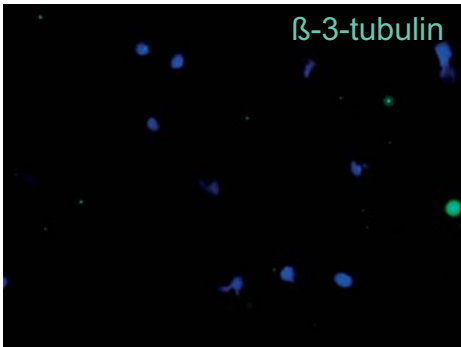
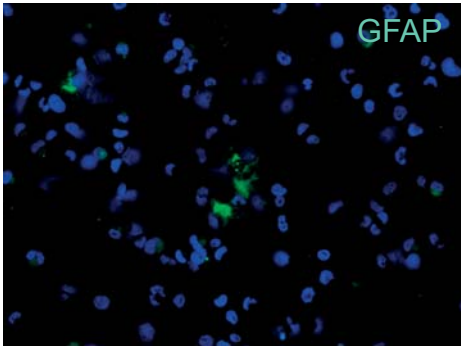
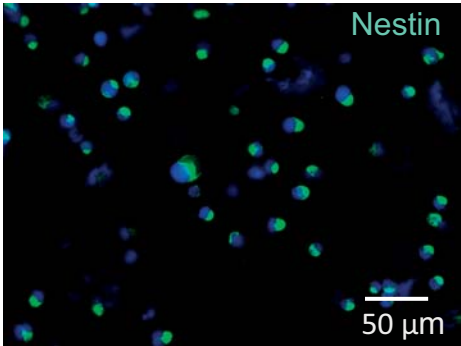


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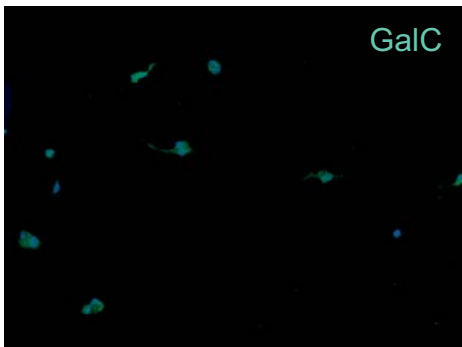
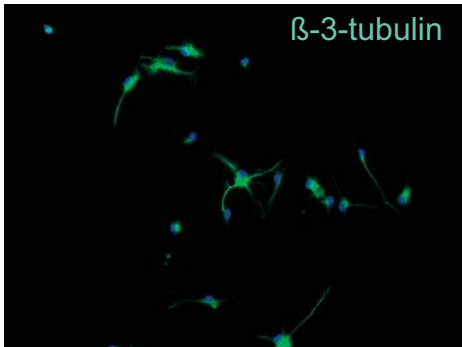
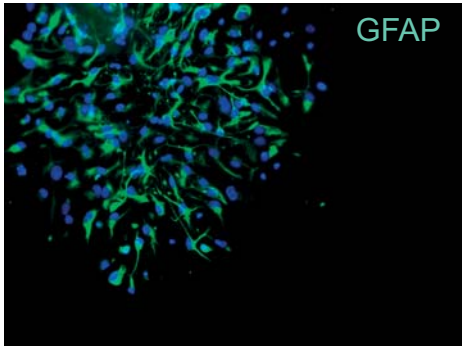
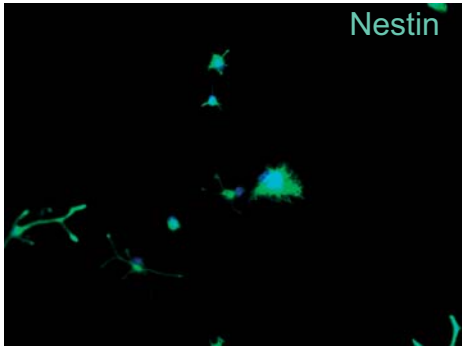


Supplementary Figure 2

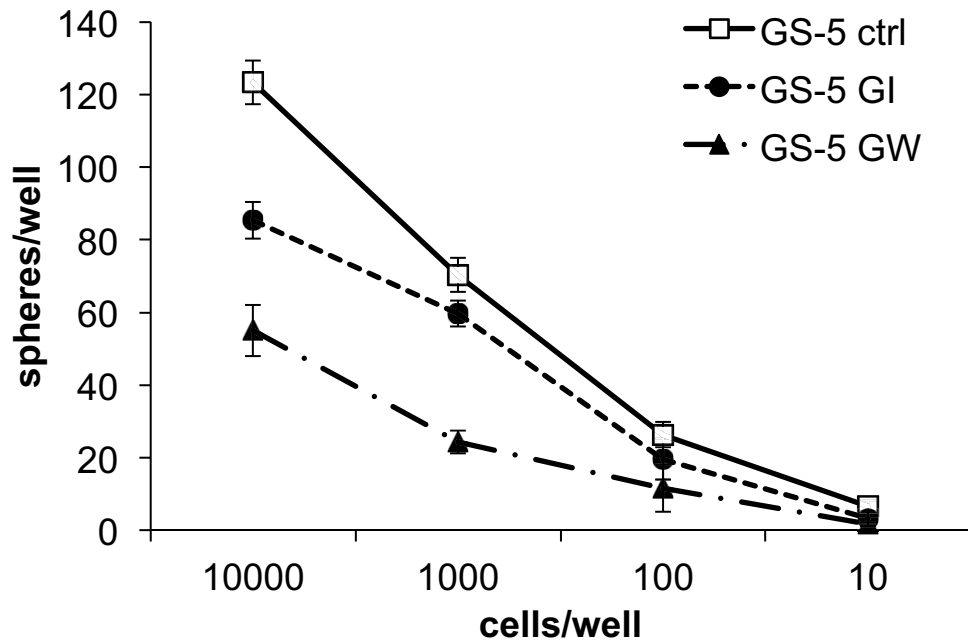
GS-9 GSC



GS-9 differentiated



Supplementary Figure 3



Supplementary materials

Supplementary Figure 1: Proliferation of GSC is inhibited through inhibition of

ADAM10 and ADAM17. 2.5×10^5 GS-7 cells were seeded in a 24 well plate in the presence of increasing concentrations of GI254023X or GW280264X (3 μ M) or of DMSO for 48 h. Viability (A) and the cell count (B) were assessed by Trypan blue staining using Vi-CELL™ XR. Viability (%) and cell count data represent mean values \pm SD from 3 independent experiments (* $p < 0.05$; student's two tailed t-test).

Supplementary Figure 2: Multipotency of GSC. (A) Cytospin samples of GS-9

GSC under stem cell culture conditions (left column) or after 8 days under differentiating culture conditions (right column) were prepared and stained for nestin, β -3 tubulin, GalC or GFAP (green), counterstaining of cell nuclei was performed with DAPI (blue). Note the scale bar in the upper left image (50 μ m).

Supplementary Figure 3: Inhibition of ADAM10 and ADAM17 compromises

sphere forming capacity of GSC. GS-5 cells were dissociated to a single cell suspension and 10, 100 and 1000 cells respectively were seeded in 96 well plates in triplicates. The cells were treated with either DMSO, GI254023X or GW280264X (3 μ M each) for 14 days. Then the number of spheres was counted in each well using a

light microscope. Results represent mean number of spheres \pm SD. One representative out of two experiments is shown.